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## **The calcium-binding protein S100A2 interacts with p53 and modulates its transcriptional activity**

Mueller, A ; Schäfer, B W ; Ferrari, S ; Weibel, M ; Makek, M ; Höchli, M ; Heizmann, C W

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**Mechanisms of Signal Transduction:  
The calcium binding protein S100A2  
interacts with p53 and modulates its  
transcriptional activity**

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Ferrari, Mirjam Weibel, Miro Makek,  
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## **The calcium binding protein S100A2 interacts with p53 and modulates its transcriptional activity.**

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Running title: Interaction of S100A2 protein with p53

Key words: S100A2, EF-hand protein, calcium, p53, head and neck squamous cell carcinoma (HNSCC)

## SUMMARY

Head and neck squamous cell carcinoma express high levels of the EF-hand calcium-binding protein S100A2 in contrast to other tumorigenic tissues and cell lines where the expression of this protein is reduced. Subtractive hybridization of tumorigenic versus normal tumor-derived mammary epithelial cells have previously identified the S100A2 protein as potential tumor suppressor. The biological function of S100A2 in carcinogenesis, however, has not been elucidated to date. Here, we report for the first time that during recovery from hydroxyurea (HU) treatment, the S100A2 protein translocated from the cytoplasm to the nucleus and co-localized with the tumor suppressor p53 in two different oral carcinoma cells (FADU and SCC-25). Co-immunoprecipitation experiments and electrophoretic mobility shift assay (EMSA) showed that the interaction between S100A2 and p53 is  $\text{Ca}^{2+}$ -dependent. Preliminary characterization of this interaction indicated that the region in p53 involved with binding to S100A2 is located at the C-terminus of p53. Finally, luciferase-coupled transactivation assays where a p53-reporter construct was used, indicated that interaction with S100A2 increased p53 transcriptional activity. Taken together, our data suggest that in oral cancer cells the  $\text{Ca}^{2+}$ - and cell cycle- dependent p53-S100A2 interaction might have a role in modulating proliferation.

## INTRODUCTION

S100A2 is a member of the subfamily of S100  $\text{Ca}^{2+}$ -binding proteins, characterized by two distinct EF-hand structural motifs. It is a homodimeric protein that upon binding of calcium undergoes a conformational change (1,2). The transduction of calcium signals in that form regulates many cellular functions such as the control of cell growth and proliferation (3), transcription (4) and p53- dependent growth arrest and apoptosis (5,6) . The S100A2 protein has been first detected in lung and kidney and is mainly expressed in a subset of tissues and cells such as breast epithelia and liver (6-10).

The S100A2 and 20 other S100 genes are located on a cluster on human chromosome 1q21, a region frequently rearranged in human cancer (6,11). Interestingly the cDNA coding for the S100A2 protein was identified as a novel tumor suppressor gene by subtractive hybridization between normal and tumor-derived human mammary epithelial cells (12). Expression studies showed that the S100A2 gene is markedly down-regulated in several tumor tissues of various origins like melanomas (13) and breast carcinoma (14). Moreover, growth factors were reported to alter the S100A2 gene expression at late G<sub>1</sub>- S-phase, indicating that S100A2 is cell cycle regulated (15). Site-specific DNA methylation of the S100A2 gene promoter region in normal versus tumorigenic breast cancer cell lines indicated repression of gene expression in tumor cells, thus suggesting a role for S100A2 in suppression of tumor cell growth and possibly inhibition of tumor progression (16).

Recently, much attention has been paid to the expression of the S100A2 gene and gene product in head and neck squamous cell carcinoma (HNSCC). In contrast to breast (14,16) and colon carcinoma (17), the S100A2 protein is overexpressed in a subset of HNSCC (18,19). Furthermore, S100A2 expression in HNSCC has been positively associated with squamous cell

differentiation and negatively with tumor grading (20). Immunolocalization studies revealed that the protein, preferably located in the nucleus in normal tissue (21-23), becomes both nuclear and cytoplasmic in tumorigenic HNSCC tissues (24).

The transcription factor p53 is overexpressed in HNSCC (25,26) and its subcellular localization correlates with tumor stage (27) and tumor progression (28). Induction of p53 transactivation activity by DNA-damage results into increase of S100A2 transcription (29). Furthermore, S100B, which is present in neuronal tissues and is associated with brain tumors (8,30-32), and S100A4, overexpressed in metastatic breast cancer cell lines (33) were recently reported to interact with p53 and this, in turn was shown to cause decrease of p53 transcriptional activity. However, although interactions between p53 and S100 proteins are of particular interest, the mechanism of S100-p53 regulated growth arrest, and in particular the role of S100A2 in carcinogenesis in HNSCC, has not been elucidated to date.

In an attempt to study the biological role of S100A2, we investigated the subcellular localization of S100A2 and p53 proteins in HNSCC cell lines synchronized with the DNA-replication inhibitor hydroxyurea (34). Next, we examined the calcium dependence of S100A2-p53 association *in vivo* using two distinct HNSCC lines as well as cells derived from breast cancer epithelia. The interaction between the two proteins was confirmed through *in vitro* pull-down assay using full-length and truncated p53 proteins. Moreover, the effect of this interaction on p53 transactivation was examined in a luciferase-coupled reporter assay. Our data provide the first insights into the regulation of p53 activity by S100A2.

## EXPERIMENTAL PROCEDURES

*DNA Constructs*— Full-length cDNA of human p53<sub>1-393</sub> and the deletion construct p53<sub>73-393</sub>, both containing a Kozak consensus start, were cloned into the mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA) by PCR employing a 5' *Bam*HI site and a 3' *Not*I site. p21-luc (in pGL2), p53<sub>Asp281-> Gly</sub> and p53<sub>1-362</sub> (lacking the S100B binding site) were gifts from Patrick Chène, Novartis, Basel and have been described previously (35,36). The full-length S100A2 cDNA was cloned as a fusion into pGEX-3X vector (Pharmacia P-L Biochemicals, Milwaukee, USA) by PCR using a 5' *Bam*HI site and 3' *Eco*RI site. All constructs were controlled by sequencing.

*Human squamous cell carcinoma tissues biopsies*—Human HNSCC tissue sections originating from patients suffering from hypopharynx- and tongue carcinoma were fixed in formalin and embedded in paraffin. The sections were stained using the following antibodies: monoclonal mouse anti-human p53-DO-1 (p53-DO-1; Santa Cruz, Biotechnology, USA), monoclonal mouse anti-human p53-1801 (p53-1801; Santa Cruz, Biotechnology, USA), polyclonal human anti-rabbit S100A2 (anti-S100A2), polyclonal human anti-rabbit S100A4 (anti-S100A4) and polyclonal human anti-rabbit S100A6 (anti-S100A6; all from Dako, Glostrup, Denmark), at a dilution of 1:25. Human tissue samples were analyzed using a wide field microscope (Leica, Switzerland), at a resolution 20x.

*Human HNSCC-Cell Lines*— FADU (originating from the hypopharynx, ATCC HTB-43) and SCC-25 cells (from a tongue, ATCC CCL-1628; kindly provided by Dr. C Decaestecker, Institute de Pharmacy, U.L.B., Bruxelles), normal breast epithelial cell line HBL-100 (HCC1187, ATCC, Manassas, VA), and H1299 lung adenocarcinoma cells (CRL-5803, ATCC, Manassas, VA) were



all grown at 37°C in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10 % FBS (fetal bovine serum), 100 IU/ml penicillin and 100 IU/ml streptomycin (complete medium). Transfections were performed using the calcium phosphate method.

*Cell Synchronization Assay and Cell Cycle Analysis*—Exponentially growing FADU, SCC-25, and HBL100 cells were treated with 2 mM HU (Fluka, Buchs, Switzerland) for 24, 28 and 20 hours, respectively, to obtain G<sub>1</sub>/S-phase arrest. Synchronized cells were released from the HU-block and subjected to cell cycle analysis. For FACS analysis, samples were collected at the indicated time points, trypsinized, washed and stained with propidium iodide (25 µg/ml) according to the manufacturer's guidelines (Cycle Test<sup>TM</sup>Plus DNA Reagent Kit, Becton Dickinson, Basel, Switzerland). Stained samples were analyzed in a fluorescence-activated cell sorter (FACS Calibur, Becton Dickinson, Basel, Switzerland) and cell cycle distribution was analyzed with WinMDI software.

*Immunofluorescence*— FADU and SCC-25 cells were grown on glass cover slips and synchronized with HU. Cells were fixed in 2% paraformaldehyde for 15 min. at room temperature, washed 4 times with PBS, permeabilized using 0.1 % Triton for 1 min and blocked in DMEM-horse serum (1%) for 1 hour at room temperature after extensive washes with PBS. Slides were incubated with the following antibodies: monoclonal anti-p53-DO1 at a dilution of 1:100 and polyclonal anti-S100A2 at a dilution of 1:500 for 1 h at 37° C. Samples were washed with PBS and incubated with the secondary CY2-and CY5- conjugated anti-mouse and the CY3-conjugated anti-rabbit antibody (Dianova, Hamburg, Germany) both at a dilution of 1:200 as described (37). Nuclear stainings were performed using DAPI. Control stainings were performed

on untreated cells and with the secondary antibody alone. Localization of the proteins was obtained with a Leica confocal microscope (DMIRE; Wetzlar, Germany).

*Western Blot*— For whole cell extracts, cells were lysed in ice-cold lysis buffer (50 mM Tris buffer (pH 7.5), 1 mM EDTA, 0.1 mM PMSF, 10 µg/ml Leupeptin, 1% Triton, 5 mM β-mercaptoethanol, 100 mM NaCl) and samples were clarified by centrifugation for 15 min at 14000 rpm in an Eppendorf centrifuge. 100µg of protein extracts were resuspended in loading buffer, heated for 5 min at 95°C and loaded onto a 4-12%- gradient SDS-PAGE gel (Invitrogen, Carlsbad, CA). Upon separation of proteins under denaturing conditions, proteins were transferred to Nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) and probed with the following antibodies: polyclonal anti- S100A2 at 1:2000 dilution, monoclonal anti-p53-DO1 antibodies at 1:5000 dilution for 1h at room temperature in TBST / 3 % milk-powder. Membranes were washed in TBST, incubated with the secondary anti-rabbit- or the anti-mouse-HRP conjugated antibody (1:10000), respectively for 1h at room temperature and exposed to ECL detection reagent (ECL, Amersham Biosciences, Buckinghamshire, UK). Protein bands were visualized on Kodak films.

*Co-immunoprecipitation*— FADU and HBL100 cell extracts were prepared as described above and precleared with Protein G-and Protein A- Sepharose beads (Amersham Biosciences, Uppsala, Sweden). Monoclonal anti-p53-DO-1 and polyclonal anti-S100A2 antibodies were coupled to Protein-G- and Protein-A-Sepharose beads, respectively, and washed with NET-80 buffer (20 mM Tris, pH=7.5, 80 mM NaCl, 1 mM EDTA) for 1 h at room temperature. The beads were again washed with NET-80 buffer and precleared cell extracts were incubated for 6 hours at 4° C in the presence of 0.1-2 mM CaCl<sub>2</sub>. Pellets were centrifuged at 11000 rpm, rinsed once with

NET-80 buffer and three times with buffer A (50 mM Tris, pH 8.0, 0.2% Triton, 500 mM NaCl), buffer B (50 mM Tris, pH 8.0, 0.1% Triton, 150 mM NaCl, 0.1% SDS) and buffer C (50 mM Tris, pH 8.0, 0.1% Triton). Western blotting was carried out as described above.

*Electrophoretic Mobility Shift Assay* — An oligonucleotide derived from the p21/WAF promoter (33) was labeled with [ $\gamma$ - $^{32}$ P]ATP (Amersham Biosciences, England). Human p53 proteins in nuclear extracts were obtained from H1299 cells after transient transfection using the calcium phosphate method. Human recombinant S100A2 protein was purified as previously described (8). Nuclear extracts were incubated in the presence of 5x gel shift binding buffer (20% glycerol; 5mM MgCl<sub>2</sub>; 2.5mM EDTA; 2.5mM DTT; 250mM NaCl; 50mM Tris-HCl pH7.5; 0.25mg/ml poly(dI-dC)) and 0.5 $\mu$ g and 1 $\mu$ g recombinant S100A2 protein. For the S100A2 interaction with p53, the reaction mix was incubated in the presence of 2 mM calcium or 5 mM EDTA for 1-2 hours at 4°C. Radiolabeled oligonucleotide p21/WAF was added and the reaction was allowed to proceed for an additional 20 min. at room temperature. The assay was terminated with 1ul of gel loading 10 x buffer (250mM Tris-HCl pH7.5; 0.2% bromophenol blue, 40% glycerol). Samples were run on 5% non-denaturing polyacrylamide gel (CleanGel™ System 25S, Pharmacia Biotech) according to the manufacturer's guidelines. The gel was dried and exposed to X-ray film at -80°C overnight.

*GST-Pulldown Assay*— The GST-S100A2 fusion construct was expressed from pGEX-3X and purified as previously described (38). p53 full-length and deletion mutants were cloned in pcDNA3 plasmid (Invitrogen Carlsbad, CA) and used to produce [ $^{35}$ S] labeled proteins (Amersham Biosciences, England) with the TNT-coupled transcription-translation system

(IVT, Promega, Madison, USA): 2 µg GST-S100A2 coupled to glutathione-Sepharose beads washed with 2 mM CaCl<sub>2</sub> were incubated with 4 µl of the IVT reaction mix and NET-80 buffer (80 mM NaCl, 20 mM Tris) for 3 hours at 4° C. Radiolabeled proteins were pulled-down, washed extensively with NET-80 buffer and separated on a 4-12 % SDS-Gel NuPAGE (Invitrogen, Carlsbad, CA). The gel was dried and exposed to X-ray film at –80 °C to visualize radiolabeled proteins.

*Transcriptional Activation Assay*— A luciferase construct driven under the control of the p21 promoter (p21-luc) was used to study the transcriptional activity of p53. H1299 cells (6 x 10<sup>5</sup>) were plated in 60-mm dishes in the presence of complete medium and transfected after 24 hours using the calcium phosphate method. The following constructs were used: p53<sub>1-393</sub>, p53<sub>73-393</sub> and p53<sub>1-362</sub> (lacking the S100B-p53 binding site) and S100A2. After 48 h of transfection, luciferase activity was measured according to the manufacturer's guidelines (Promega, Madison, USA). Transfection efficiency was normalized using β-galactosidase activity. Results are the mean of three independent experiments. Statistical significance was evaluated using the Student T-test.

## RESULTS

*p53 and S100 proteins are differentially localized in human HNSCC tissue biopsies*—S100 and p53 display a characteristic nuclear and cytoplasmic staining in tumor biopsy sections (Fig.1) originating from the tongue (*upper panel*) and the hypopharynx (*lower panel*). Wild-type p53 predominantly accumulated in the nucleus in both tissue sections but was found in the cytoplasm of sections of the tongue (*upper panel, a*). S100A2 was expressed in the cytoplasm and diffusely in the nucleus in sections of the tongue (*upper panel, c*) as well as the hypopharynx (*lower panel,*

c). Compared to S100A2, S100A4 (*upper and lower panel, d*) and S100A6 (*upper and lower panel, e*) were both exclusively expressed in the cytoplasm of HNSCC tissue biopsies. The staining for the S100B protein was negative (*upper and lower panel, f*). To further investigate the evidence on the cytosolic localization of S100A2 and p53, which are normally nuclear proteins (14,22), we examined cell lines derived from HNSCC tissues (FADU and SCC-25 cells).

*Cell cycle-dependent localization of S100A2 in human FADU and SCC-25 cells*—To assess whether transition through the cell cycle affects S100A2 subcellular localization, FADU and SCC-25 cells expressing both endogenous S100A2 and p53 proteins were treated with HU. HU blocks ribonucleoside diphosphate reductase and leads to rapid depletion of deoxyribonucleotide pool, thereby arresting the cells at the G<sub>1</sub>/S-boundary. Immunofluorescence staining of untreated FADU cells showed that p53 and S100A2 were predominantly localized in the nucleus, whereas cytoplasmic staining for both proteins was evident in SCC-25 cells (Fig.2, *panel A, B*). The merged images indicated partial co-localization of p53 and S100A2 in the nucleus in FADU but mainly cytoplasmic co-localization in SCC-25 cells. Treatment of FADU or SCC-25 with 2 mM HU for 24 or 28 hours, respectively, resulted in synchronization at G<sub>1</sub>/S as shown by flow cytometric analysis (Fig.3, *panels A,B*). Under these conditions, p53 and S100A2 co-localized in the nucleus and the cytoplasm in both cell lines (Fig.2, t=0, *panel C, D*). Upon HU removal (t=1), cells synchronously moved into S-phase (Fig.3, *panel A,B*) and this was characterized by translocation of p53 to the nucleus in FADU and SCC-25 cells. At this time point, S100A2 translocated to the nucleus in both cell lines (Fig. 2, *panel C, D*). At 8 and 10 hours, respectively, after release from the HU block (t=2), cells were mostly in G<sub>2</sub> phase (Fig. 3, *panels A, B*) and both p53 and S100A2 proteins were exclusively present in the nucleus. During transition through the next cell cycle, at 12 and 16 hours after release from the HU block (t=3), p53 and S100A2

were redistributed to the cytoplasm where they co-localized. Finally, in FADU cells, 24 hours after release from the HU block ( $t=4$ ), p53 and S100A2 staining returned to the pattern displayed in untreated cells.

FACS analysis of FADU and SCC-25 cells indicated that the less differentiated SCC-25 had a longer cell cycle as compared to the former (Fig.3, *A, B*). Taken together these data show cell cycle-dependent shuttling of p53 and S100A2 in FADU and SCC-25 cells with co-localization in the nucleus at late S/G2-phase.

*Endogenous S100A2 and p53 Interact in FADU and HBL100 Cells in a Calcium Dependent Manner*— Co-localization of p53 suggested that the two proteins may physically interact. To substantiate this finding, we performed co-immunoprecipitation experiments using total extracts of FADU cells. Precleared FADU cell lysates were immunoprecipitated with p53-DO-1 antibody in the presence of increasing calcium concentrations or 5mM EDTA. Proteins were resolved by SDS-PAGE and detected by Western blot analysis with anti-S100A2 (Fig.4A). The results showed that S100A2 co-immunoprecipitated with p53 in the presence of calcium concentrations higher than 0.1 mM (*lanes 3-5*). In contrast, no interaction between p53 and S100A2 could be detected in the presence of a calcium chelator (*lane 7*) or at low calcium concentration (*lane 2*). Identical results were obtained when immunoprecipitations (IP) were performed with S100A2 antibody and p53 was detected with p53-DO-1 antibody (Fig. 4B). To rule out the possibility that the signal given by the IgG-heavy chain of the antibody used in IP could be erroneously interpreted as p53, we performed control-IP using anti-S100A2 antibody in the absence of cell extract. The results indicated that no protein band at the level of ~50 kD could be detected when using antibody p53-DO-1 for Western Blotting (*panel B, D, lane 1*). Western blotting of S100A2 confirmed the efficiency of the antibody used for IP (Fig. 4, *panel C, E*).

To support the evidence obtained from FADU cells, we employed HBL100 cells that originate from breast cancer tissue and, like FADU cells, express wild-type p53 and S100A2 (Fig. 4D). As shown in Fig. 3 panel C, HBL100 cells could also be synchronized with HU. Precleared HBL 100 cell lysates were incubated with anti-S100A2 antibody under the same conditions used for FADU cells. Also in the case of HBL100 cells, the interaction between p53 and S100A2 was readily observable though at slightly higher calcium concentrations (*lane 5*) than in FADU cells. As observed in the latter, addition of 5mM EDTA during IP completely abrogated this interaction (*lane 7*). Taken together, these findings suggest that the interaction between p53 and S100A2 is  $\text{Ca}^{2+}$ -dependent and occurs in cell lines expressing the two proteins, independent of the tumor type from which the cell originate.

*S100A2 Binding to p53 Affects its DNA Binding Activity*—To characterize the physiological consequences of S100A2 binding to p53, we examined p53 DNA binding activity (Fig. 5). To this end, we performed electrophoretic mobility shift using nuclear extracts from H1299 cells and a labelled oligonucleotide containing the p53-consensus sequence of the p21 promoter. Since H1299 cells lack both p53 and S100A2, they were transiently transfected with the p53<sub>1-393</sub> construct (*lane 1*). Addition of 0.5  $\mu\text{g}$  recombinant S100A2 protein to H1299 nuclear extracts resulted in supershift of the oligonucleotide-p53 complex in the presence (*lane 4*) but not in the absence of 2 mM calcium (*lane 3*). Addition of 5mM EDTA to the p53-S100A2 complex completely reverted the effect of calcium (*lane 5*).

Furthermore, we tested DNA-binding in the presence of increasing amounts of S100A2. Previous reports have shown that increasing amounts of S100B reduce the intensity of p53 complex binding to its responsive element (30,31,39-41). Similarly, in the presence of 1 $\mu\text{g}$  S100A2 and 2 mM calcium, the intensity of the S100A2-p53 complex was clearly reduced (*lane 7*). Again,

calcium was essential for the induction of the supershift and the latter could be reversed by 5 mM EDTA (*lane 8*). Ectopic expression of p53 proteins in transiently transfected H1299 cells was controlled by Western blot analysis (Fig. 5B). These results show that the  $\text{Ca}^{2+}$ -dependent interaction between p53 and S100A2 facilitates binding of p53 to its responsive element.

*S100A2 interacts with wild-type and the C-terminus of p53*—We next examined the domains required for the interaction of S100A2 with p53. We performed *in vitro* GST-pull down assay (Fig.6) using *in vitro* translated and [ $^{35}\text{S}$ ]-labeled p53<sub>1-393</sub>, p53<sub>73-393</sub> and p53<sub>1-362</sub>. Recombinant GST-S100A2 proteins were immobilized on glutathione-Sepharose beads, incubated with the *in vitro* translated p53 proteins and the eluted complexes were separated on SDS-PAGE. Under these conditions, full-length p53 protein was pulled down in the presence of 2 mM calcium whereas the interaction was almost completely abolished in the presence of 5 mM EDTA (Fig.6, *upper panel lanes 3, 4*).

Deletion of the p53 transactivation domain (p53<sub>73-393</sub>) did not affect the calcium-dependent interaction between the C-terminal domain of p53 and recombinant S100A2 ( Fig.6, *middle panel, lane 3*). In contrast, deletion of the C-terminal domain, where the S100B-binding site was mapped (residue 367-388, (1), resulted in complete loss of the binding to GST-S100A2 (Fig. 6, *lower panel, lane 3*). These data showed that the p53 region necessary for binding to S100A2 is located in the C-terminus of p53.

*S100A2 Modulates Transcriptional Activity of p53* —To test the functional consequences of the S100A2-p53 interaction, we performed transient transfections in H1299 cells, which are deficient in p53 and S100A2. Transfection of the p53 wild-type expression vector resulted in a ~ 19.5 fold activation of transcription of the p21 reporter gene construct (Fig. 7A, *lane 3*) whereas



transfection of S100A2 had no effect on the luciferase reporter (*lane 2*). Co-transfection of the p53 expression vector with S100A2 resulted in a further ~7.2 fold increase of p53-dependent transcriptional activation. Titration experiments showed that maximal p53 activation was obtained at 100 ng S100A2 DNA (*lane 4*) with a reduction of transcriptional activity to almost basal level at 500 ng S100A2 (*lane 5*). Furthermore, we employed a p53 construct containing a mutation in the N-terminal region (Asp281-> Gly) that caused an overall reduction of activity, while displaying little induction of the p21-reporter construct (~1.3 fold, Fig.7, B, *lane 3*). Co-transfection with S100A2 resulted in the same extent of increase in transactivation observed with wild-type p53. The effect was maximal at 100ng of S100A2 (~3.5 fold) with a less pronounced induction at 500ng S100A2 (~1.3 fold, *lanes 4, 5*). These results indicated that S100A2 was able to activate p53 transcriptional activity in a statistically significant manner.

To further verify that the far C-terminal domain of p53 (residues 362-393) was necessary for the observed induction of p53-dependent transcription by S100A2, we transiently transfected H1299 cells with the p53<sub>1-362</sub> deletion construct (Fig. 7C).

p53<sub>1-362</sub> displayed an overall lower transactivation activity as compared to the full-length protein but was nevertheless able to activate the reporter construct (~ 4.2 fold, *lane 3*). Interestingly, the transcriptional activation of p53 was slightly reduced at 100 ng S100A2 (~0.5 fold, *lane 4*) as well as 500 ng S100A2 DNA (~0.6 fold, *lane 5*). These results indicate that S100A2 is able to activate the transcriptional activity of wild-type p53 but lack of the oligomerization domain of p53 abrogates this function. The p-values in the corresponding experiments indicate that there is a statistical significance of the transcriptional activation of wild-type and mutant p53 by S100A2.

## DISCUSSION

The S100A2 protein belongs to the calcium binding, EF-hand protein family of S100 proteins and has attracted interest due to the fact that it is localized to the nucleus (22), it is downregulated in various tumor tissues (24,42,43) and it is presumably a potential tumor suppressor gene (12,16). Site-specific methylation of the S100A2 gene promoter region and transcriptional activation of the S100A2 promoter by p53 (29) further support the hypothesis on involvement of this protein in carcinogenesis. The tumor suppressor p53 is a key regulator of the cell cycle and triggers apoptosis in response to stress (44). Stability and cellular localization of p53 is regulated by interaction with HDM2 (45) and its transactivation activity is modulated by cell type and tissue-specific co-factors (41).

Based on our previous findings that the S100A2 is localized to the nucleus (22), we investigated possible physical and functional interactions with p53 in HNSCC tissues and cell lines (FADU and SCC-25) that express both p53 (25,46,47) and S100A2 (18,48). We found that the S100A2 protein was localized in the cytoplasm with focal nuclear staining in HNSCC primary tumor tissues whereas S100A6 and S100A4 were exclusively in the cytoplasm (Fig.1). On the contrary, S100B was undetectable in tissue staining. p53 was present in nuclei as well as in the cytoplasm. This pattern of p53 and S100A2 staining was reflected in studies performed on cell lines derived from HNSCC tumors. Predominantly nuclear co-localization of p53 and S100A2 was found in FADU, whereas SCC-25 cells displayed both cytosolic and nuclear staining. Several studies have shown that wild-type p53 accumulates in the cytoplasm where it is rapidly degraded in an HDM-2 dependent manner. Cytoplasmic sequestration of p53 has been described in a variety of primary tumor tissues such as neuroblastoma (49), colon cancer (50,51) and presumably melanoma (52). Changes of the subcellular localization of p53 in these tumors have been associated with inhibition of the p53 tumor suppressive function and, as a consequence, with poor prognosis (51).

Overexpression of p53 is an early event in the carcinogenesis of HNSCC and correlates with increased recurrence, poor prognosis and poor survival rate (53,54). Members of the S100 protein family have been described to enhance nuclear accumulation of p53 (32). Moreover, a recent report showed disruption of the p53 tetramerization equilibrium by S100A4 and S100B that lead to accumulation of p53 in the nucleus (55). To examine whether p53 and S100A2 localization and interaction was cell-cycle dependent, we considered cells synchronized with HU. This drug is widely used for cell synchronization and the reversibility of its effect makes it particularly suitable to use in cell culture. Moreover, HU is used in the treatment of thrombocythaemia (56). Similar to HU, Fluorouracil (FU) applied in chemotherapy of colon and HNSCC inhibits DNA synthesis by thymidine starvation resulting in growth arrest and cytotoxicity (57). We found that during transition through the S- and G<sub>2</sub>-phases that follow synchronization with HU, p53 and S100A2 translocated to the nucleus and co-localized. After transition through M-phase, the two proteins were redistributed to the nuclear and cytosolic compartments, as observed in non-synchronized cells. The mechanism of the p53-S100A2 complex shuttling between the cytoplasm and the nucleus remains to be determined.

We further investigated the physical interaction of endogenous S100A2 and p53 proteins in FADU and HBL 100 cells (Fig. 4) in the presence or the absence of calcium. Titration experiments indicated that p53 and S100A2 proteins interacted only at calcium concentrations higher than 0.5mM in FADU and higher than 2 mM in HBL100 cells. The property of S100A2 to bind calcium with lower affinity as compared to other S100 family members may explain the results obtained. The conditions employed in our assay closely reflected the elevated intracellular calcium concentrations caused by deregulated calcium homeostasis that has been reported to occur in human tumors (58). Moreover, interaction between S100B and p53 has also been studied using high calcium concentrations (1-2 mM, (30,31,39,59,60). Furthermore, our data showed

that the  $\text{Ca}^{2+}$ -dependent interaction of S100A2 with p53 involved the C-terminal domain of p53 (Fig. 6). These region encompasses the tetramerization domain and contains the S100B binding site (residue 367-388) (1,61,62). Given the high sequence similarity between the S100 family members, it is likely that S100A2 interacts at the same binding site as S100B binding. Finally, our studies indicate that S100A2 activates the transcriptional activity of full-length p53 (Fig. 7). A p53 mutant at the residue that is a common hot spot for mutation in numerous tumors of the colon and breast (63), and that causes reduction of p53 transactivation activity responded similarly to wild-type p53 when co-transfected with S100A2. Furthermore, our finding that transactivation of a reporter construct by the deletion mutant p53<sub>1-362</sub> was reduced in co-transfection experiments with S100A2 (Fig. 7C) supports our conclusion of a potential S100A2 binding motif in the C-terminus of p53.

S100A2 protein expression is high in tumors of the oral cavity but not in breast and neuroblastoma. Prognosis of S100A2 positive HNSSC than of negative tumors is generally better. S100A2 has been used as a tumor marker (19). The interaction of S100A2 and p53 that we report in this study, is of particular interest since, contrary to other S100 family members, S100A2 activates p53 transcriptional activity, presumably helping to restore p53 function in growth arrest and apoptosis. The recent report of an interaction between S100A2 and p63 (64), a p53 family member substantiates the importance of our findings.

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## FOOTNOTES

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## ABBREVIATIONS

The abbreviations are used: FADU/SCC-25: oral epithelial carcinoma cells; HBL100: Human breast epithelia cell line; HU: Hydroxyurea; IP: Immunoprecipitation; GST: Glutathion-S-transferase; IVT: *in vitro* trans-lated; HNSCC: head and neck sqamous cell carcinoma; DMEM: Dulbecco's Modified Eagle Medium.

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**Fig. 1 Immunohistochemical localization of S100A2 and p53 proteins in the cytoplasm and the nucleus in HNSCC tissues.** Expression of S100 and p53 proteins in malignant HNSCC tissues, originating from the tongue (*upper panel, a-f*) and the hypopharynx (*lower panel, a-f*) using the following antibodies: anti-p53-1801 (*a*), anti-p53-DO-1 (*b*), anti-S100A2 (*c*), anti-S100A4 (*d*), anti-S100A6 (*e*) and anti-S100B (*f*) antibodies. The arrows indicate positive cytoplasmic and the arrow heads positive nuclear staining of p53 and S100A2. S100A4 and S100A6 proteins are mainly in the cytoplasm. Note the negative staining of S100B. (x 20 objective, H&E, scale bar 20  $\mu$ m).

**Fig. 2 Subcellular localization of S100A2 and p53 in HU synchronized FADU and SCC-25**

**cells.** Localization of p53 (green) and S100A2 proteins (red) in untreated FADU cells (*panel A*) and SCC-25 cells (*panel B*). Arrow heads indicate nuclear and cytoplasmic staining of p53 and S100A2 in both cell lines. Synchronization of FADU cells with 2mM HU and staining of anti-p53-DO1 (green) and anti-S100A2 (red) antibodies at the indicated time points (*panel C*). Detection of p53 (blue) and S100A2 proteins (red) in SCC-25 cells, treated with 2 mM HU and stained at the corresponding time points (*panel D*). DNA staining was performed with DAPI. Scale bar: 40  $\mu$ m.

**Fig. 3. Cell cycle analysis of FADU and SCC-25 cells synchronized with HU.** FADU (*panel A*), SCC-25 (*panel B*) and HBL100 cells (*panel C*) synchronized with HU (2mM) for 24, 28 and 20 hours respectively were released at the indicated times in complete medium and stained with propidium iodide prior to flow cytometric analysis.

**Fig. 4. S100A2 physically interacts with p53 in a  $\text{Ca}^{2+}$ -dependent manner.** *A*, FADU cell extracts subjected to immunoprecipitation (IP) with monoclonal anti-p53-DO-1 to p53 in the presence of calcium (0.1 mM, 0.5 mM, 1mM and 2mM) or a calcium chelator (5 mM EDTA). Western blotting was performed with polyclonal anti-S100A2 antibody as described under “Materials and Methods”. *B*, IP of FADU cell extract with a polyclonal anti-S100A2 under conditions described in *A*, followed by detection of p53 with monoclonal anti-p53-DO-1. *C*, analysis of the extent of S100A2 precipitated from FADU cell extracts. *D*, *E* IP of HBL100 extracts with polyclonal anti-S100A2 antibody, followed by detection of p53 and S100A2 as indicated in *B* and *C*. Control- IP with p53- DO-1 to p53 or anti-S100A2 antibody in the absence of cell extracts are shown in *lane 1* of each panel. Anti-p53-DO-1 were used at a dilution of 1:5000, anti-S100A2 at a dilution of 1:2000 and the corresponding secondary-HRP-coupled antibodies at a dilution of 1:10000.

**Fig. 5. Electrophoretic mobility gel shift of p53 and S100A2 interacting in a  $\text{Ca}^{2+}$  - dependent manner.** Nuclear extracts (6  $\mu\text{g}$ ) of p53 positive (+) H1299 cells were incubated with the oligonucleotide derived from the p21/WAF promoter labeled with  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  and increasing amounts of human recombinant S100A2 proteins (0.5/ 1  $\mu\text{g}$ ) in the presence (+), (*lane 4,5,7*) or absence (-), (*lane 6,8*) of calcium. Specific competitor (unlabeled p21/waf oligo) 500 x (*lane 2*).

**Fig. 6. S100A2 interacts with wild-type and the C-terminal domain of the p53 protein *in vitro*.** Human recombinant S100A2 was produced in *E.coli* as a fusion protein with GST, conjugated to glutathione-Sepharose beads and incubated with *in vitro* translated (IVT) and [<sup>35</sup>S]-labeled full-length p53 (p53<sub>1-393</sub>, *upper panel*), p53 C-terminus (p53<sub>73-393</sub>, *middle panel*) and p53 deletion construct (p53<sub>1-362</sub>, *lower panel*) lacking the S100B-binding site (1) in the presence (2 mM calcium) or absence (5 mM EDTA) of calcium. *Upper panel, lane 1*: GST-S100A2 protein (control); *lane 2*: p53<sub>1-393</sub> (input); *lane 3*: GST-S100A2 incubated with 20% IVT p53<sub>1-393</sub> and 2mM calcium; *lane 4*: GST-S100A2 incubated with 20% IVT p53<sub>1-393</sub> and 5mM EDTA. *Middle panel, lane 1*: GST-A2 fusion protein (control); *lane 2*: GST-S100A2 incubated with p53<sub>73-393</sub> (input); *lane 3*: GST-A2 incubated with input 20% IVT p53<sub>73-393</sub> and 2mM calcium; *lane 4*: GST-A2 incubated with 20% input IVT p53<sub>73-393</sub> and 5mM EDTA. *Lower panel, lane 1*: GST-A2 fusion protein (control); *lane 2*: GST-S100A2 incubated with p53<sub>1-362</sub> (input); *lane 3*: GST-A2 incubated with 20% input IVT p53<sub>1-362</sub> and 2mM calcium; *lane 4*: GST-A2 incubated with 20% input IVT p53<sub>1-362</sub> and 5mM EDTA.

**Fig. 7. Luciferase reporter assay to measure the transcriptional activity of wild-type, mutated and truncated p53 in the presence of S100A2.** H2199 cells were transfected with the p21-responsive luciferase reporter containing binding sites for p53 and the p53<sub>1-393</sub> wild-type construct (*A*), mutated p53<sub>Asp281-> Gly</sub> (*B*) and truncated p53<sub>1-362</sub> (*C*) con-comitantly with increasing amounts of full-length S100A2.

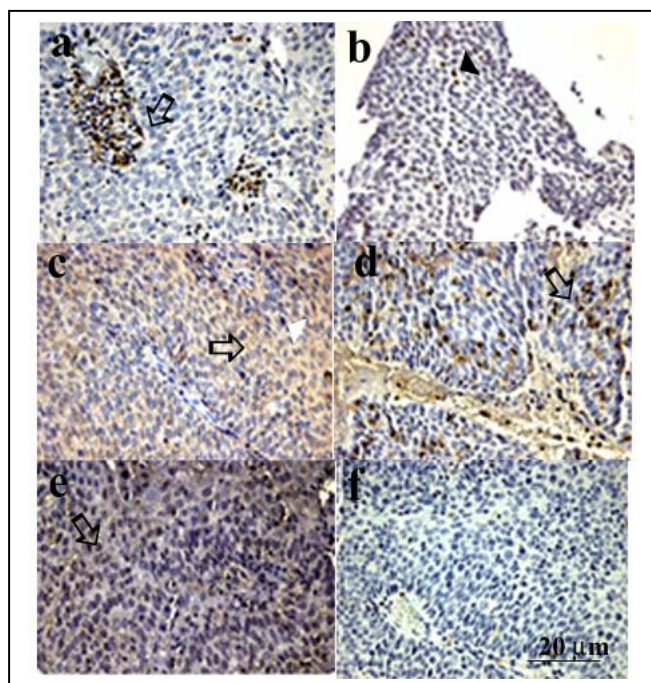
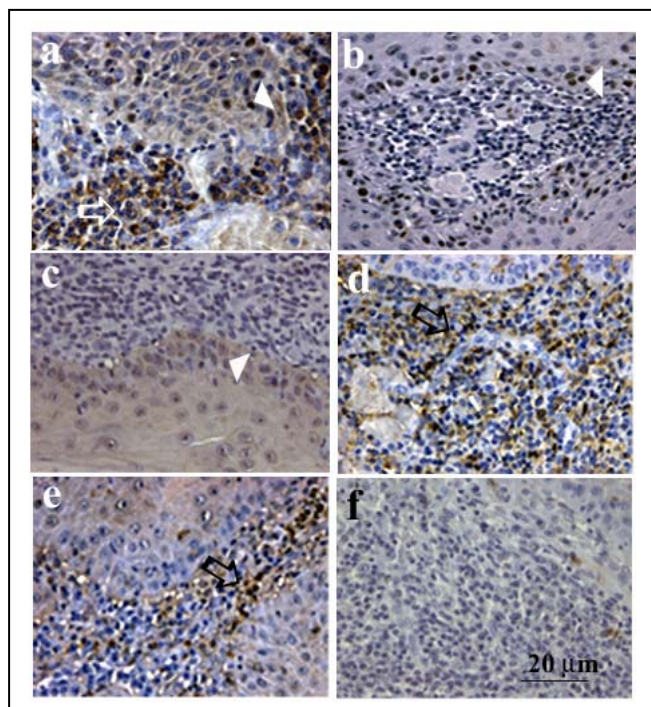
*A*, lane 1: 1,5 µg p21- luc only; lane 2: 1,5 µg p21- luc with 100 ng S100A2 only; lane 3: 1,5 µg p21- luc with 100 ng p53<sub>1-393</sub>; lane 4: 1.5 µg p21- luc with 100 ng p53 p53<sub>1-393</sub> and 100 ng S100A2; lane 5: 1,5 µg p21- luc with 100 ng p53<sub>1-393</sub> and 500 ng S100A2. p-value: \*3→4: 0.029

*B*, lane 1: 1,5 µg p21- luc only; lane 2: 1,5 µg p21- luc with 100 ng S100A2 only; lane 3: 1,5 µg p21- luc with 100 ng p53<sub>Asp281-> Gly</sub>; lane 4: 1.5 µg p21- luc with 100 ng p53<sub>Asp281-> Gly</sub> and 100 ng S100A2; lane 5: 1,5 µg p21- luc with 100 ng p53<sub>Asp281-> Gly</sub> and 500 ng S100A2. p-value: \*3→4: 0.0065

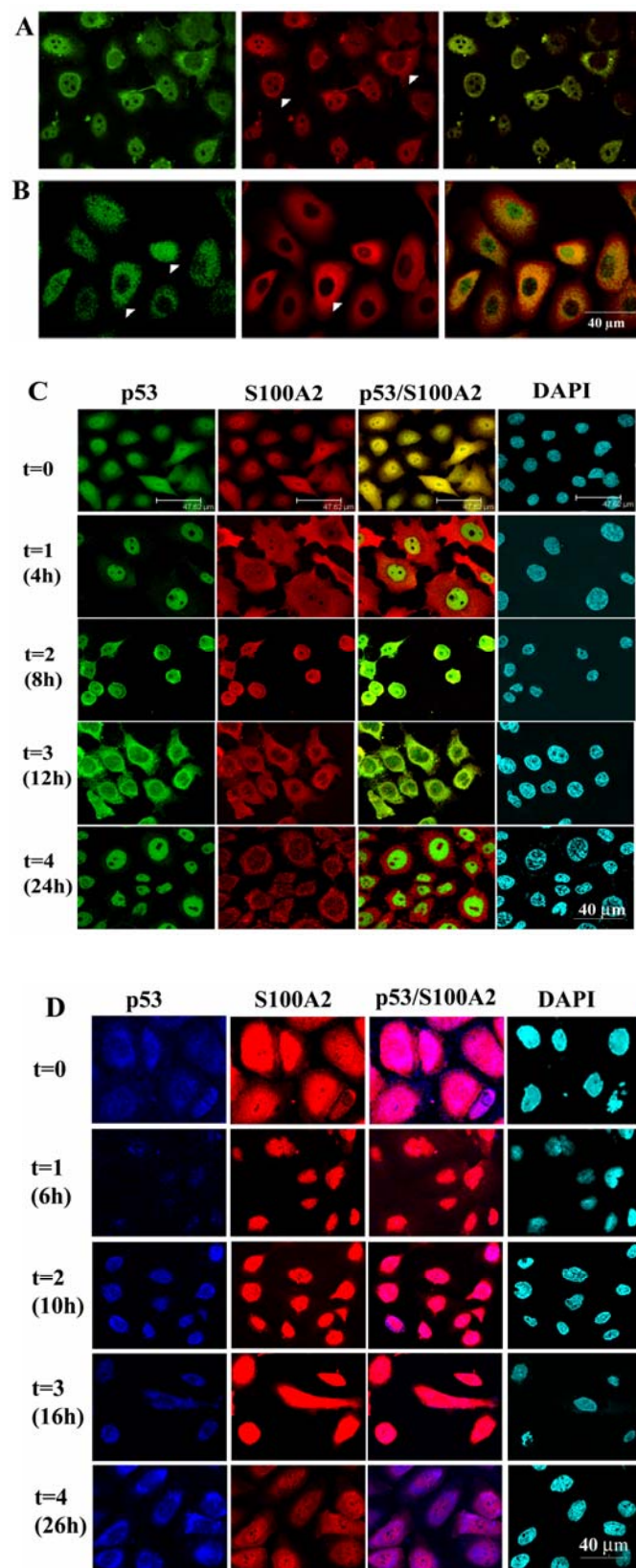
*C*, lane 1: 1,5 µg p21- luc only; lane 2: 1,5 µg p21- luc, 100 ng S100A2 only; lane 3: 1,5 µg p21- luc with 100 ng p53<sub>1-362</sub>; lane 4: 1.5 µg p21- luc with 100 ng p53<sub>1-362</sub> and 100 ng S100A2; lane 5: 1,5 µg p21- luc with 100 ng p53<sub>1-362</sub> and 500 ng S100A2. p-value: \*3→4: 0.0073

Results are representative of three independent experiments and normalized as described under “Materials and Methods.”

**Fig. 1**





**Fig. 2**

**Fig. 3**

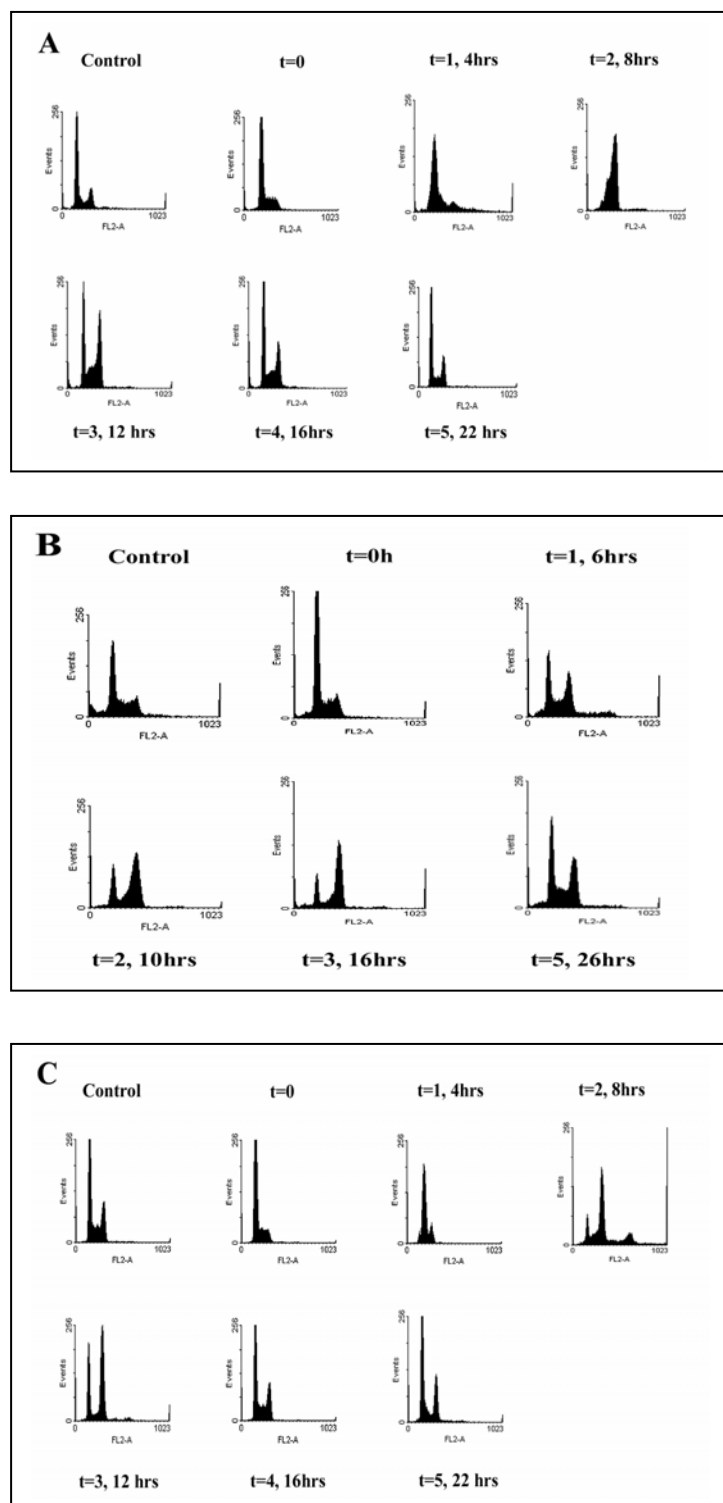
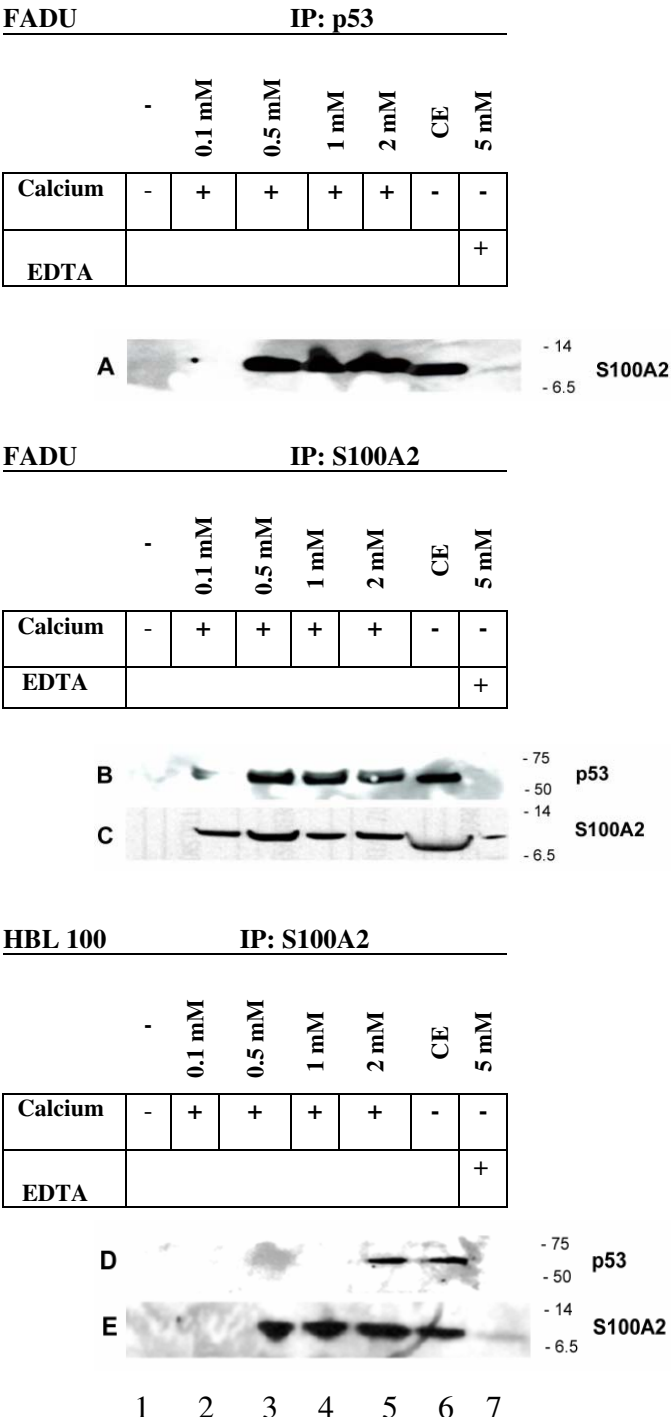
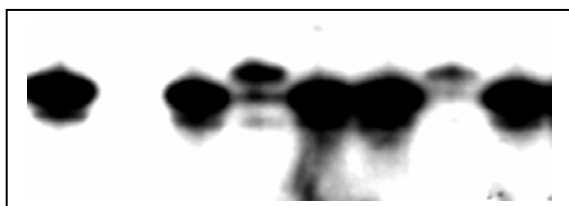
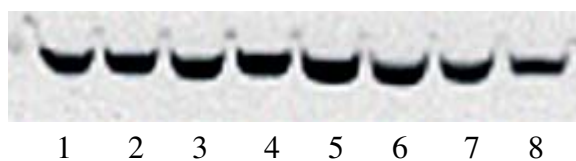


Fig. 4

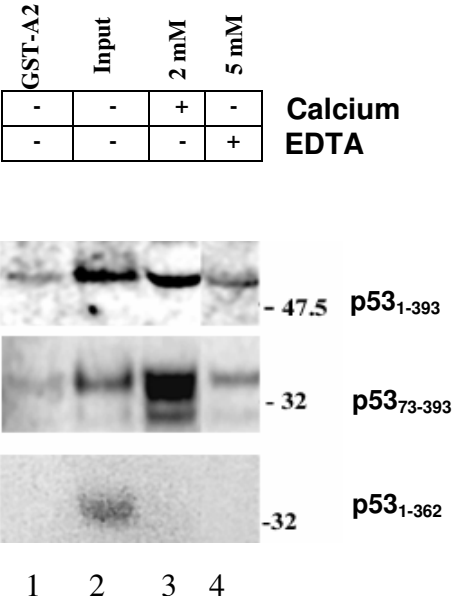


**Fig. 5****A**

<b>H1299 p53</b>	+	+	+	+	+	+	+	+
<b>Specific competitor</b>	-	+	-	-	-	-	-	-
<b>Calcium (2mM)</b>	-	-	-	+	+	-	+	+
<b>EDTA ( 5mM)</b>	-	-	-	-	+	-	-	+
<b>S100A2 ( 0.5 µg)</b>	-	-	+	+	+	-	-	-
<b>S100A2 ( 1 µg )</b>	-	-	-	-	-	+	+	+

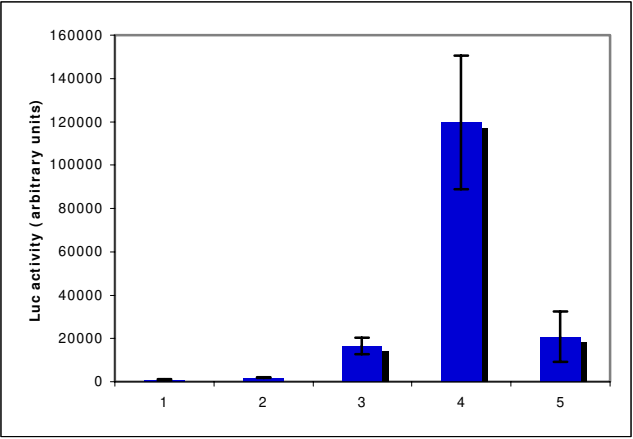
**B****H1299: p53**

**Fig. 6**



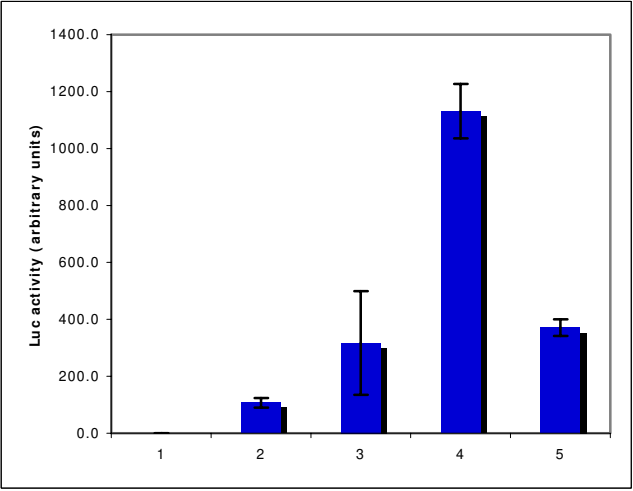
**Fig. 7**

**A**



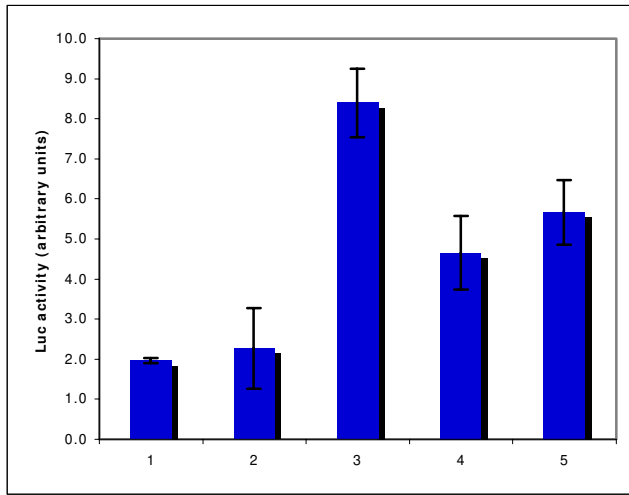
p21-luc	+	+	+	+	+
p53 <sub>1-393</sub>	-	-	+	+	+
S100A2	-	+	-	+	+
β-gal	+	+	+	+	+
pcDNA 3	+	+	+	+	+

**B**



p21-luc	+	+	+	+	+
p53 <sub>Asp281-</sub>	-	-	+	+	+
Gly	-	+	-	+	+
S100A2	-	+	-	+	+
β-gal	+	+	+	+	+
pcDNA 3	+	+	+	+	+

C



<b>p21-luc</b>	+	+	+	+	+
<b>p53<sub>1-362</sub></b>	-	-	+	+	+
<b>S100A2</b>	-	+	-	+	+
<b>β-gal</b>	+	+	+	+	+
<b>pcDNA 3</b>	+	+	+	+	+